
Dugway Proving Ground
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Chemical & Biological
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Report Documentation Page

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Inactivation of *Bacillus anthracis* Spores in Drinking Water by Mixed Oxidant Solution

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Background

- Soldiers in some tactical scenarios do not have access to quartermaster potable water supplies.
- When calcium hypochlorite is used as a water disinfectant, a high free available chlorine concentration (FAC) is required to kill *B. anthracis* spores.
- Hypochlorite is toxic at levels around 190 mg/kg body weight.

Background, cont.

- Water treated with calcium hypochlorite at a level to kill anthrax spores is neither potable nor palatable.

Chemistry

- FAC is the concentration of Cl_2 + HOCl + OCl^- (expressed as Cl_2) and is the most chemically reactive and biocidal form of chlorine commonly used for disinfection
- Hypochlorous acid (HOCl) is the predominant form of FAC at pH < 7.2.
- Optimum biocidal effectiveness of FAC occurs at pH 6.0 to 7.5.

Chemistry, cont.

- At high pH HOCl dissociates primarily to hypochlorite ion ($OCl^- + H^+$).
- OCl^- is about 1/100 as effective a biocide as is HOCl, but still effective.
- HOCl at pH < 4 forms chlorine gas (Cl_2).
- Combined chlorines are formed from the reaction of FAC with nitrogenous compounds such as ammonia and urea.

Chemistry, cont.

- Reactions with ammonia or amino group-containing organic compounds produce chloramines and organic chloramines.
- Chloramines are formed in a step-wise manner as FAC is added resulting in
  - Monochloramine (NH_2Cl)
  - Dichloramine ($NHCl_2$)
  - Trichloramine (NCl_3)

Chemistry, cont.

- Chloramines are weak oxidizing agents and biocides relative to both HOCl and OCl^-.
- One source suggests that “monochloramine may only have 5% of the disinfecting power of FAC on a time/concentration basis.”

Mixed Oxidants

- Produced by the electrolysis of sodium chloride solution
- Complete characterization of the molecular species present has not been possible.
- Characteristics of mixed oxidant activity suggest species are present in addition to HOCl and OCl^- (ie. FAC)

Mixed Oxidant Advantages

- Rapid disinfection rates
- Removal of biofilms from surfaces of water distribution systems and treatment works
- More stable chlorine residual

Disinfection Pen

- MIOX Corporation
- Los Alamos Technical Associates funded by DARPA to investigate technology for single soldier use
- Life Sciences Division tasked to provide independent evaluation using simulants and vaccine strains.

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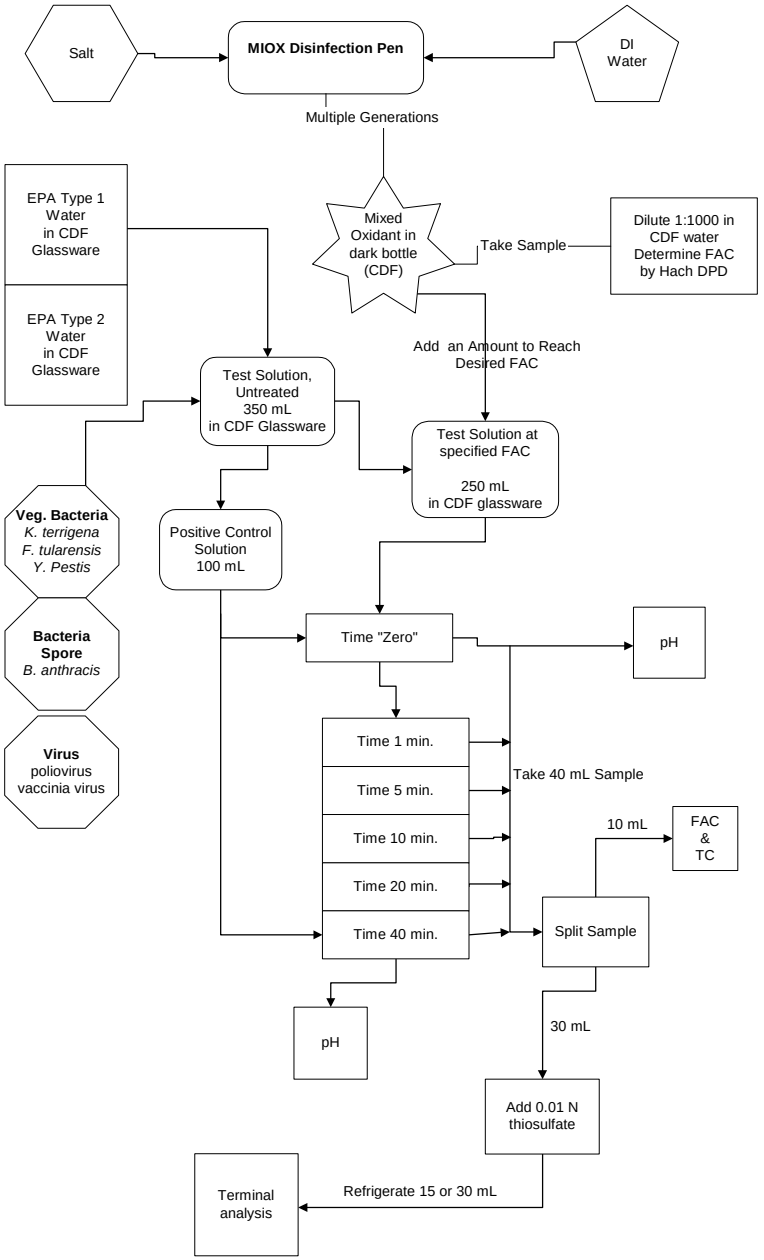
Initial Dugway Studies

- EPA Type 1 Water
  - Free of any chlorine or other disinfectant residue
  - pH 6.5 to 8.5
  - Total organic carbon 0.1 - 5.0 mg/L
  - Turbidity 0.1 to 5 NTU
  - Temperature 20±5°C
  - Total dissolved solids (sea salt) 50 - 100 mg/L



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Initial Studies, cont.

- *Bacillus anthracis* strain Sterne
  - spores
  - washed
- Looked for a 6 log reduction
- Challenge dose of 1E7 cfu/mL
- Details of test published as WDTC Document WDTC-IR-01-047

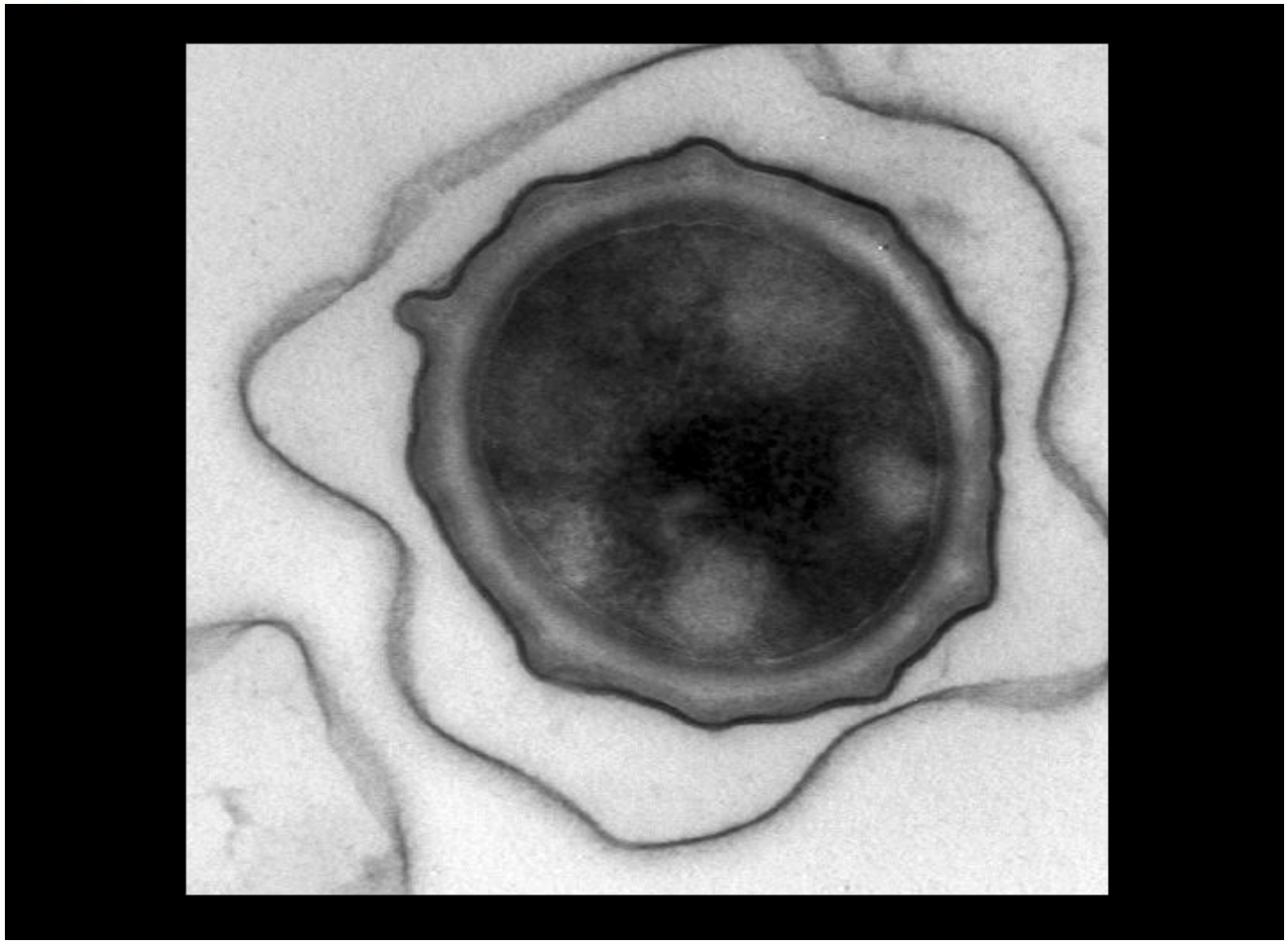
Initial Studies, cont.

- Result: The 7 log cfu/mL challenge dose was killed in 40 minutes when treated with MOS at a FAC of 160 mg/L
- Analysis: It would take a soldier 40 MOS generation cycles of the pen to produce the required does in a 1-liter canteen.
- Conclusion: Impractical

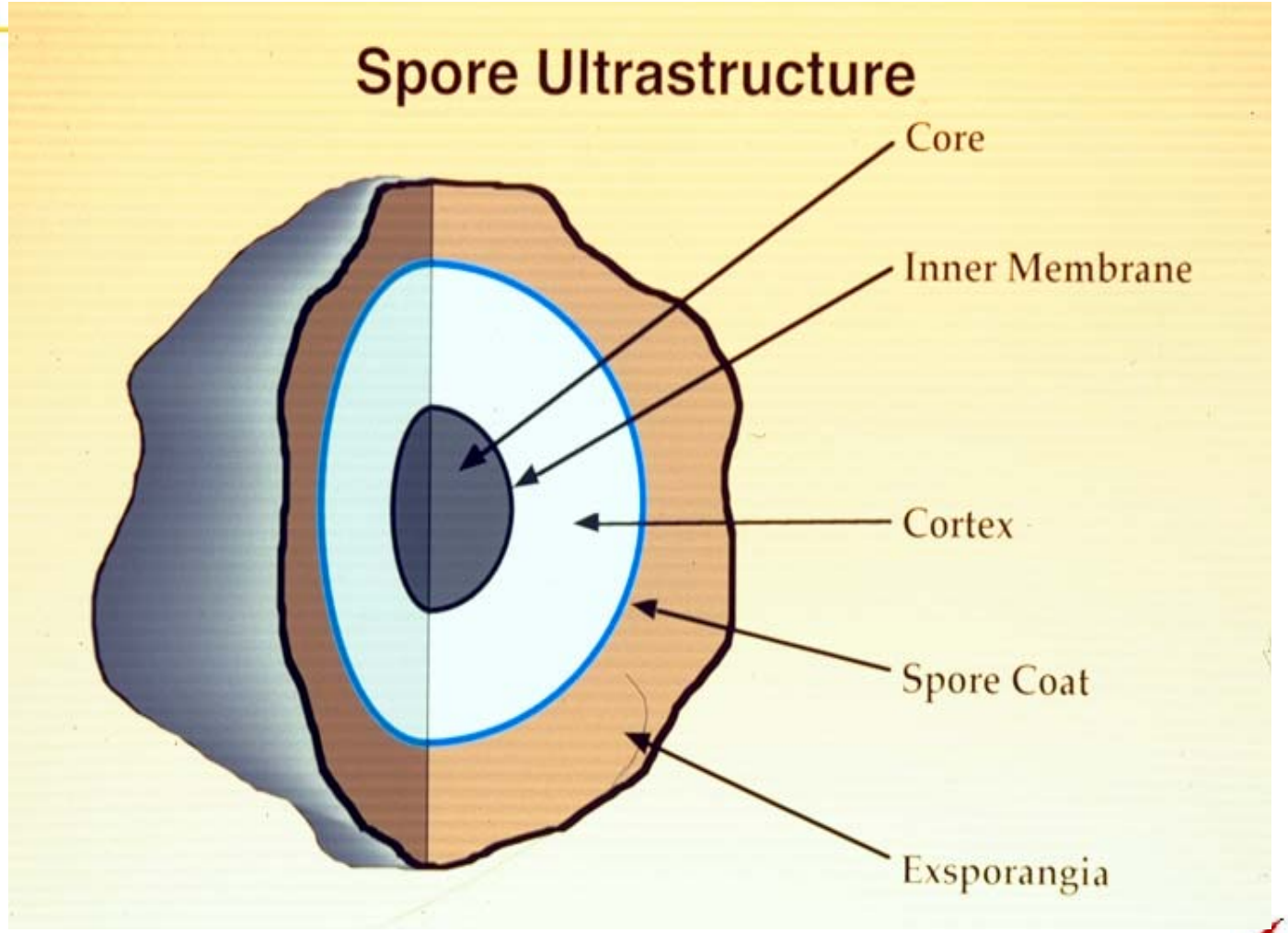
Follow-on Studies

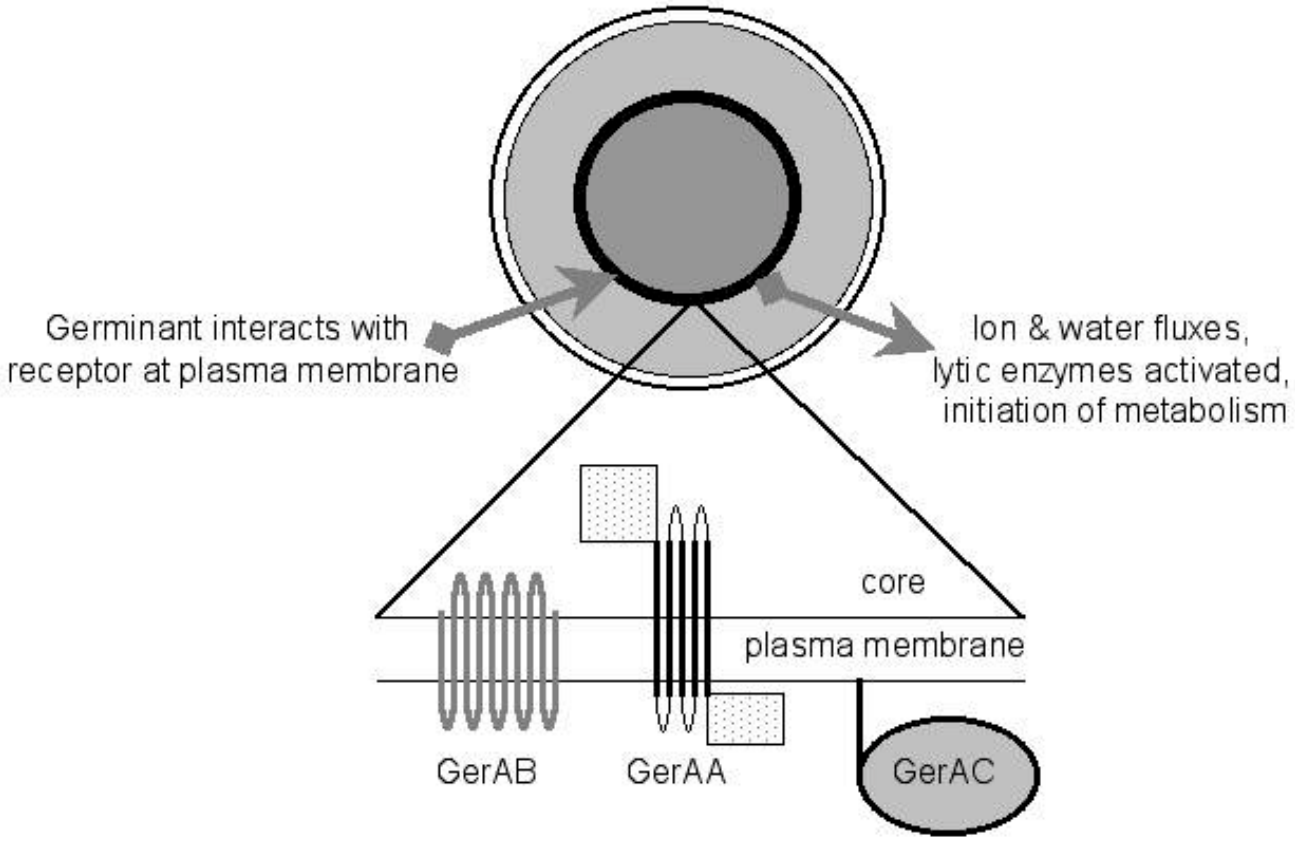
- *B. anthracis* vegetative cells known to be sensitive to chlorine inactivation
- Pre-treat water with a germinant solution
- Phil Hanna (Univ. of Michigan) suggested
  - 10 mM L-alanine
  - 1 mM serine
  - 1 mM inosine

Anthrax Spore Ultrastructure



Anthrax Spore Ultrastructure




Germinant interacts with
receptor at plasma membrane
Ion & water fluxes,
lytic enzymes activated,
initiation of metabolism
core
plasma membrane
GerAB
GerAA
GerAC

Germinant Method A

- Used sterile tap water (no chlorine demand)
- Add Sterne spores to concentration of 1E6 cfu/mL
- Add germinant, incubate 30 minutes
- Add MOS to calculated FAC of 160 mg/L
- Plate samples at 1, 5, 10, 15, 25, 35, 45, & 60 minutes; incubate overnight

Germinant + MOS Results

- Sterne spores would germinate using concentrations 1/10 of those recommended:
  - 1 mM L-Alanine
  - 0.1 mM Serine
  - 0.1 mM Inosine
- Our measure of germination being sensitivity to MOS.
- Germination results consistent with those previously published

Method A Results, cont.

- Chlorine demand, germinant ~ 155 mg/L over 25 minutes
- Spores killed in 25 minutes after adding MOS at a final FAC decreasing to ~5.
- Significant increase in killing power of MOS on germinated spores
- Not unexpected

Germinant Method B

- Same as Method A except that germinant and MOS were added to the spore suspension within less than 10 seconds of each other

Method B Results

- Same as from Method A
- Spores killed in 20-30 minutes without a significant (<10 sec) incubation in germinant.
- **Unexpected result**
- No biochemical explanation evident
- Obvious that the majority of the FAC was being combined with germinants to form organic chloramines almost instantaneously.

Organic Studies B

- The preceding results suggested another set of experiments.
- Looking at our results not as a function of germination but as a function of organic chloramine species and the N content of the germinants.

Organic Chloramine Studies, cont.

- Theoretical framework from water treatment chemistry
  - A FAC ratio of 5 x N mass concentration (ie. 5 mg FAC/mg N) + 25% should generate organic monochloramine with negligible amounts of organic di- and trichloramine
  - The 25% additional FAC satisfied non-N oxidant demand, determined separately.

Organic Chloramine Studies, cont.

- A FAC ratio of 7 x N mass concentration + 25% should generate a combination of organic chloramines, NOT primarily monochloramine
- A FAC ratio of 7.6 x N + 25% should drive the reaction to breakpoint where the N is released from the chloramines as N_2 and FAC is reduced to Cl^- (ie. both FAC and chloramines reduced in concentration in the water)

Organic Chloramine Experiments

- Prepare germinant solution such that N = 21 mg/L in 1E6 cfu/mL Sterne spore suspension
- Dose with 5 FAC x N or 7 FAC x N
- React for 30 minutes at room temperature
- Dose with 7.6 x N to cause breakpoint
- Plates samples at 1, 5, 15, 25, 40, 60, & 90 minutes (no thiosulfate added)

L-Ala, Ser, & Inosine: 5 x N

Time (min)	FAC	TC	cfu/mL
1	40	79	5.9E3
5	21	51	1E2
15	14	30	0

L-Ala, Ser, & Inosine: 7 x N

Time (min)	FAC	TC	cfu/mL
1	17	32	9E1
5	11	23	3E1
15	7	17	0

Organic Chloramine Conclusions

- Both the 7 x N and 5 x N doses against the complete germinant kill the spores within 15 minutes.
- The 5 x N dose has a flatter reaction curve.
- No clear difference between a solution presumably consisting of monochloramines versus a chloramine cocktail.

Inorganic Chloramine Study

- Substitute ammonium chloride as the nitrogen source because chloramine generation from this molecule simpler in concept.
- Believed this experiment would be a negative control: assumed organic germinants possess some inherent quality required to explain previous results

Inorganic Chloramine Experiment

- Ammonium chloride (NH_4Cl) substituted for germinant solution at same nitrogen concentration (21 mg/L)
  - Preston & Douthit (J Gen Microbiol, 1984) described its co-germinant effects on *B. cereus*.
- A sample was also taken after 15 min treatment and before the second or "breakpoint" 7.6 x N dose was added.

NH_4Cl: 5 x N

Time (min)	FAC	TC	cfu/mL
1	9	47	0
5	8	22	0
15	7	17	0

NH_4Cl: 7 x N

Time (min)	FAC	TC	cfu/mL
1	5	10	0
5	5	9	0
15	4.5	7.3	0

15 Minute Samples

- 5 x N = < 10 cfu/mL
- 7 x N = 0 cfu/mL

Inorganic Chloramine Conclusions

- A solution of chloramines derived from an inorganic source is capable of inactivating Sterne spores within 15 minutes.
- The effect was evident at 4 mg/L NH_4-N (down from 21 mg) but not at 2 mg/L suggesting a very pronounced activation threshold.
- Monochloramines are probably the most active species.

Inorganic Conclusions, cont.

- At 4 mg/L NH_4-N, the MOS dose required (38 mg/L FAC) is practical using the latest version of the Disinfection Pen.
- Preston and Douthit (1983, 1988) conducted experiments with *B. cereus* in which the germinant activity of NH_4Cl was studied in the presence of Ala and other known co-germinants.
- Our results suggest that Sterne spores are sensitive to NH_4Cl alone, probably at some point downstream from the recognition points for organic co-germinants

Inorganic Conclusions, cont.

- This effect has been confirmed in preliminary experiments at Phil Hanna's lab in the absence of MOS.

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